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A Solution and Solid State Study on 2-Hydroxypropyl-β-Cyclodextrin Complexation with Hyodeoxycholic Acid

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Abstract. Hyodeoxycholic acid (HDCA) is a 6-α dihydroxylated natural bile acid capable of preventing gallstone formation by reducing the bile cholesterol saturation. However, any attempt to enrich the bile acid pool with HDCA have failed owing to the poor solubility of the molecule. To resolve the bioavailability problems, the complexation of HDCA into the HPβCD cavity was studied in solution (solubility methods, ^{13}C- and ^{1}H-NMR spectroscopy and circular dichroism) and in the solid state (IR spectroscopy, X-ray diffractometry and thermal analysis). According to the results, the HDCA inclusion took place with 1 : 1 stoichiometry. The influence of different preparation methods of the solid complex was evaluated for its potential use in appropriate pharmaceutical formulations to improve the bioavailability of HDCA.

Key words: hyodeoxycholic acid; 2-Hydroxypropyl-β-cyclodextrin; Inclusion complex; Solubility studies; ^{13}C- and ^{1}H-NMR spectroscopy; Circular dichroism; IR spectroscopy; X-ray diffractometry; Thermal analysis; Dissolution studies

1. Introduction

Oral administration of individual bile acids lead to their differential increase in bile; the enrichment is strong with chenodeoxycholic acid and poor with hyodeoxycholic acid (HDCA) (Figure 1) [1, 2]. In animals HDCA administration is

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able to prevent and dissolve cholesterol gallstones [3, 4], but in humans this bile acid shows low bioavailability because of its urinary excretion in glucuronated form [2, 5]. The solubility of HDCA in water and in saline is very low [5, 6]: total bile acid concentration, ionic strength, pH and temperature influence the amount of bile acid existing in solution and suitable for absorption. The physicochemical properties in a model bile make HDCA able to dissolve a high concentration of cholesterol in the liquid crystalline phase [7] as demonstrated in prairie dogs [4]. Therefore, the tauroconjugated form of HDCA has been synthesised to improve its water solubility and bioavailability in humans [8].

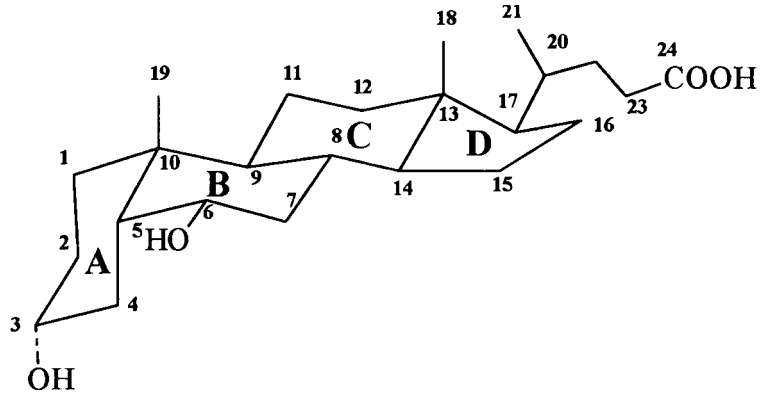


Figure 1. Hyodeoxycholic acid (HDCA) (3α, 6α-dihydroxy-5β-cholan-24-oic acid).

To resolve bioavailability problems related to poor solubility and the low dissolution rate of drugs in biological fluids, a different approach could consist in the inclusion of the drug molecules into the cavity of the cyclodextrin (CD) derivatives [9, 10]. Among numerous other water soluble βCD derivatives of pharmaceutical interest (sulfobutylether βCD, randomly methylated βCD and the branched βCDs), 2-hydroxypropyl-β-cyclodextrin (HPβCD) exhibits excellent water solubility [11] allowing drug complexes having high solubility to be obtained. The inclusion complexes between HPβCD and several bile acids (cholic, chenodeoxycholic, and ursodeoxycholic acids) have been previously prepared and characterised both in solution and in the solid state [12–14]. Oral administration to healthy volunteers of appropriate tablets containing the ursodeoxycholic acid complex produced higher bioavailability with respect to that of commercial tablets containing the uncomplexed drug [15]. As the haemolytic effect of the bile acids decreased owing to the complexation, the i.v. administration of HPβCD/bile acid complexes was proposed in the treatment of patients submitted to liver transplantation [16].

On the basis of these researches, the aim of this work was to study the complexation of HDCA into the HPβCD cavity in order to gain information on the feasibility of oral administration of the bile acid in liver diseases. In addition, the

aim of this work consists in the evaluation of different preparation methods of the solid complex for its potential use in appropriate pharmaceutical formulations.

2. Experimental

2.1. MATERIALS

Hyodeoxycholic acid (HDCA) (3α, 6α-dihydroxy-5β-cholan-24-oic acid; Mol. Wt 392.6) (Sigma Chemical Co., St. Louis, MO, USA) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) (average molar substitution 0.6, average Mol. Wt. 1380) (Aldrich Chemical Co., Milwaukee, WI, USA) were used to prepare the solid inclusion complex. Deionized water, prepared by Milli-Q Plus (Millipore, Bedford, MA, USA) was used throughout. All the solvents (analytical grade, Carlo Erba, Milan, Italy) and products were used as received from the manufacturers without further purification.

2.2. ANALYTICAL METHOD

The concentration of HDCA in the samples was determined by HPLC [17], using a liquid chromatographic system equipped with a 112 Solvent Delivery Module and a 166 UV/Vis Programmable Detector Module (Beckmann, Fullerton, CA, USA). Chromatographic separation was performed on a Lichrospher 100 RP 18 column (240 mm long, 4.6 mm i.d.; 5 μm particles size) (Merck, Darmstadt, Germany) with a mobile phase composed of methyl alcohol-0.02 M sodium acetate in water (80 : 20, w/w); the pH was adjusted to 4.3 with phosphoric acid. 20 μL volumes were isocratically eluted (flow rate: 1 mL min^{-1}) at room temperature. Quantitative detection of HDCA was performed at a wavelength of 210 nm using deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid) (Sigma Chemical Co.) as internal standard. A standard curve with concentrations ranging from 0.2 to 15 mg mL^{-1} was linear, with a correlation coefficient (mean $\pm$ SD) of 0.999 $\pm$ 0.001 (n = 7).

2.3. SOLUBILITY STUDIES

Solubility studies were carried out according to Higuchi and Connors [18]. An excess amount of HDCA (250 mg; 0.64 mM) was added to 5 mL of water containing different concentrations of HPβCD (0–120 mM). The suspensions were shaken in 10 mL screw-capped vials at 30 strokes min^{-1} at 25 $\pm$ 2°C. When equilibrium had been reached (after about 2 days), the content of each vial was filtered through a cellulose nitrate membrane filter (pore size 0.45 μm; Sartorius, Göttingen, Germany). The filtered solution was then analysed by HPLC to determine the HDCA concentration. All the data are the average of at least three determinations (relative standard deviations were within 5%).

2.4. CIRCULAR DICHROISM

Circular dichroism spectra were recorded at room temperature using a model J-710 automatic recording circular dichrograph (Jasco, Tokyo, Japan) in the range between 200 and 280 nm using HDCA to HPβCD ratios from 1 : 0.5 to 1 : 10 (M/M) in methanol solution. Methanol was chosen as the solvent to reach a suitable concentration of HDCA (5.4 mM) for recording the CD spectra. A cylindrical fused quartz cell of 0.5 cm pathlength was used for circular dichroism measurements. The values for HDCA/HPβCD spectra are given in molar ellipticity (deg cm^2 dmol^{-1}).

2.5. PREPARATION OF THE PHYSICAL MIXTURE

The physical mixture was prepared at a 1 : 1 (HDCA : HPβCD) molar ratio by simple dry mixing of exactly weighed amounts of the two components, adopting the geometric dilution method.

2.6. PREPARATION OF THE INCLUSION COMPLEX

The inclusion complex was prepared at a 1 : 1 molar ratio of HDCA to HPβCD. Typically, 1.38 g (1 mM) of HPβCD was dissolved at room temperature in 50 mL of water to which 30 mL of ethyl alcohol containing 392 mg (1 mM) of HDCA was added. To obtain the evaporated inclusion complex, the solution was evaporated immediately after its preparation under vacuum in a rotary evaporator (model R-114; Büchi, Buchs, Switzerland) at a temperature of 45°C. To obtain the lyophilised inclusion complex, the solution was cooled immediately after its preparation to −18°C for 1 h (Shell Freezer, Edwards, Crawley, UK) and then freeze-dried at 2 mbar and −40°C for 24 h (Liovac GT2; Leybold-Heraus, Hanau, Germany).

2.7. MORPHOLOGICAL ANALYSIS

The morphology of the HDCA crystals, HPβCD, the physical mixture and the solid inclusion complexes was determined by observation on a scanning electron microscope (XL 40; Philips, Eindhoven, The Netherlands). The samples were mounted on aluminium stubs (TAAB Laboratories Equipment, Berks, England) using double sided sticky tab (TAAB Laboratories Equipment) and vacuum coated with gold for 60 sec (Polaron E-500; Balzers Union, Liechtenstein).

2.8. ^{13}C- AND ^{1}H-NMR STUDIES

^{13}C data were obtained at 303 K using an AMX-400 WB spectrometer (Bruker, Rheinstetten, Germany) operating at 100.61 MHz, on 30 mM CD_3OD solutions. ^{13}C δ values refer to internal $^{13}CD_3OD$ set to 49.3 ppm. Typical parameters for ^{13}C-NMR ^{1}H-decoupled spectra were: 0.3 Hz/pt resolution, 5s relaxation delay,

45° read pulse, 512 scans. Exponential multiplication was applied before Fourier transformation.

2.9. THERMAL ANALYSIS STUDIES

DSC curves were recorded on a Perkin-Elmer DSC-4 differential scanning calorimeter equipped with a computerised data station. Indium (99.99%) (Perkin-Elmer, Norwalk, CT) (m.p. 156.6; ΔH_f 28.45 Jg^{-1}) was used to check the instrument. All samples (5 mg about) were heated in crimped aluminium pans (Perkin-Elmer) at a scanning rate of 5°C min^{-1} using dry nitrogen flow (30 mL min^{-1}).

2.10. X-RAY DIFFRACTOMETRY

X-ray diffraction patterns were recorded using a PW 1050/25 powder diffractometer (Philips, Eindhoven, The Netherlands) using a voltage of 40 kV and a current of 20 mA for the generator, with Cu anode material. The wavelengths of the Ni filtered radiation was α_1 = 1.54178 Å. The diffractograms were recorded from 3°(2Θ) to 40°(2Θ) at an angular speed of 1° (2Θ) per minute using 1-0.1-1 slits.

2.11. IR SPECTROSCOPY STUDIES

IR spectra were recorded using an Infrared Fourier Spectrometer (model IFS 113v; Bruker) employing potassium bromide disks prepared at a pressure of 400 kg cm^{-2} (model M, Carver, Menomonee Falls, WI, USA).

2.12. DISSOLUTION STUDIES

Dissolution studies were carried out using USP XXIII apparatus 4 (Dissotest CE-1; Sotax, Basel, Switzerland) in 100 mL of water at a flow rate of 25 mL min^{-1}. All experiments were carried out at a temperature of 37 $\pm$ 0.2°C using either 150 mg of HDCA crystals or an equivalent amount of the bile acid in the physical mixture or in the solid inclusion complexes (evaporated inclusion complex and lyophilised inclusion complex). Solution samples were withdrawn at fixed time intervals and the drug concentration in the solution was determined as reported above. All experiments were run in triplicate.

3. Results and Discussion

3.1. SOLUTION STUDIES

The phase solubility diagram of HDCA in aqueous HPβCD solutions was plotted according to Higuchi and Connors (Figure 2) [18]. The solubility of HDCA increased linearly as the concentration increased. Therefore, the solubility curve could be classified as type A_L. The increase in solubility can be attributed to the

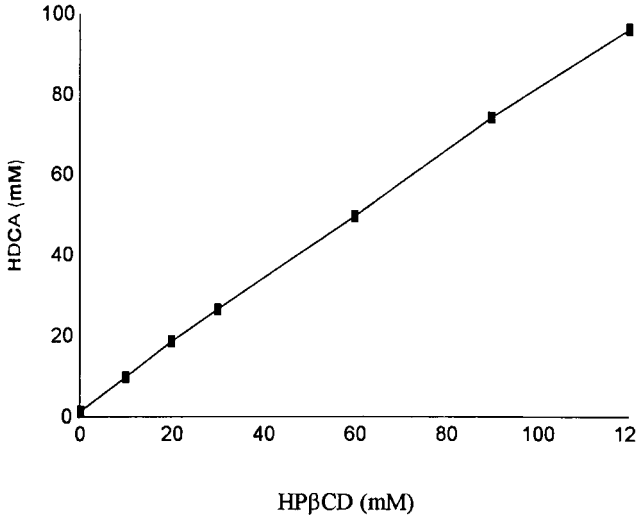


Figure 2. Phase-solubility diagram of hyodeoxycholic acid with 2-hydroxypropyl-β-cyclodextrin in water at 25 $\pm$ 2°C.

formation of an inclusion complex between HDCA and HPβCD characterized by having a greater solubility than that of HDCA alone. As the slope of the solubility curve is characterized by a slope less than 1, it can be assumed that the stoichiometry of the 1 : 1 inclusion complex is equimolar. The apparent stability constant of the 1 : 1 inclusion complex (K) was calculated from the straight line of the diagram according to the following equation:

$$K = S/[Cs(1 - S)]$$

where Cs (the intercept) is the solubility of HDCA in the absence of HPβCD and S denotes the slope of the straight line.

The value of K was found to be 1820 $\pm$ 178 M^{-1}.

The CD spectrum of HDCA exhibits a negative band at about 210 nm owing to the chiral centers present in the bile acid molecule (Figure 3), whereas no bands were observed in the spectrum of HPβCD (not reported in the figure). After interaction with HPβCD, which possesses a chiral cavity, HDCA shows a circular dichroism band at the same wavelength. However, the intensity of the negative band decreases as the HDCA/HPβCD ratio decreases. This behaviour can be related to the perturbation of the electronic transitions of the guest molecule caused by inclusion into the asymmetric cavity of cyclodextrin [19]. Therefore, the sign of the induced CD band is related to the spatial position of the asymmetric centre of the perturbed chromophore. Thus a negative CD band indicates an equatorial disposition, while the positive band indicates an axial disposition of the molecules electric dipole moment with respect to the z-axis of the cyclodextrin [20]. In our opinion, the reduction of the negative intensity of the induced circular dichroism

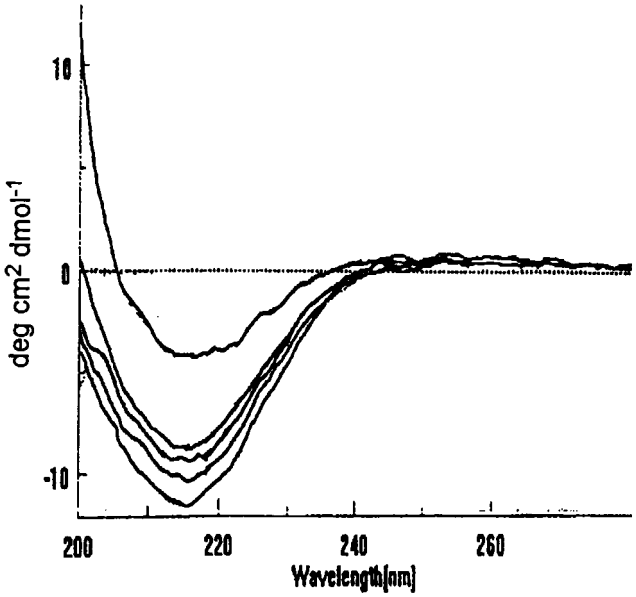


Figure 3. CD spectra in methyl alcohol. (HDCA concentration 0.0054 M). From bottom to top: HDCA; HDCA/HPβCD (1 : 0.5, M/M); HDCA/HPβCD (1 : 1, M/M); HDCA/HPβCD (1 : 2, M/M); HDCA/HPβCD (1 : 10, M/M).

band following the complexation could indicate that the electric dipole moment in the chromophore (HDCA) is axial to the long axis of the HPβCD.

The stoichiometry of the inclusion complex is confirmed by ^{1}H NMR spectroscopy. In fact, a molar ratio HPßCD : HDCA equal to 1 : 1 is observed in the ^{1}H NMR spectrum in D_2O solution. It is to be noted that HDCA signals are lost in the noise in the ^{1}H NMR spectrum obtained from a saturated HDCA solution in the same solvent and under the same conditions, due to the low water solubility of the guest.

Information on the geometry of the inclusion complex were obtained from a ^{13}C NMR study in CD_3OD solution. Previous studies on bile acid/HPβCD complexes [12, 14] have shown that, for these systems, the analysis of ^{13}C-NMR complexation shifts of the guest molecules is an easy way to derive information on complexation. In fact, the complexity of the ^{13}C and ^{1}H-NMR spectra of the HPβCD utilised (which is a complex mixture of positional isomers) prevents the accurate detection of shifts induced by complexation on the host molecule. On the other hand, the ^{13}C-NMR spectrum of HDCA is simpler than its ^{1}H-NMR counterpart and ^{13}C chemical shifts are intrinsically more sensitive than the ^{1}H shifts to the molecular environment. The 24 lines of the ^{13}C-NMR spectrum of HDCA in CD_3OD solution were assigned, together with all proton signals, through 2D-NMR ^{1}H,^{13}C inverse-detected (HMQC [21], HMBC [22]) and ^{1}H-^{1}H homonuclear [COSY [23]) experiments. The data obtained compare well with those reported in the literature

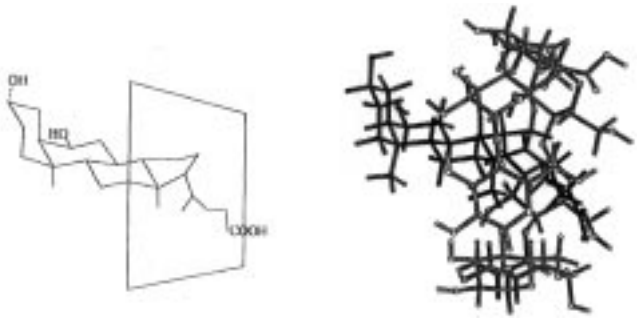


Figure 4. Schematic representation of the inclusion of hyodeoxycholic acid in cyclodextrins.

for glycosidic conjugates of HDCA [24]. An analogous procedure was utilized to attribute HDCA signals in the HDCA/HPβCD complex. ^{13}C data are reported in Table I which shows that complexation affects a well delineated region of HDCA: the D ring and the C-17 side chain. Furthermore, the first two carbons of the side chain are negatively shifted whereas the others are progressively shifted to lower fields, except C-17 and C-20 as a consequence of steric compression. These results are similar to those reported for ursodeoxycholic and chenodeoxycholic acid and are consistent with the insertion of the guest into the cyclodextrin torus from the side chain [25]. A schematic representation of the inclusion of hyodeoxycholic acid in cyclodextrins is reported in Figure 4.

For the sake of completeness, the ^{1}H-NMR chemical shifts were also analysed in free and complexed HDCA. Trends indicating the site of complexation can hardly be extracted from ^{1}H complexation shifts which are homogeneously distributed. The ^{1}H complexation shifts are all lower than +0.08 ppm (H-15α) and are all positive or zero except one of the two H-22 protons and both the two H-23 protons which are shielded in the complexed compared with the free HDC ($-$0.03 ppm).

3.2. SOLID STATE STUDIES

From the SEM microphotographs of the physical mixture, both the HDCA crystals and the HPβCD particles can be identified (Figure 5a). The bile acid crystals showed an elongated feature with a dimension ranging from a few microns up to 150–200 μm, whereas the HPβCD particles have an amorphous structure of spheroid shape with dimensions of about 5 μm. Both inclusion complexes (evaporated inclusion complex and lyophilised inclusion complex) are amorphous products in which no HDCA crystals or HPβCD particles appear (Figures 5b and 5c). According to the preparation method, the morphology of the inclusion complexes is quite different. The evaporated inclusion complex showed a more compact structure and larger dimensions (Figure 5b), whereas the lyophilised inclusion complex is formed by the aggregation of small particles (Figure 5c).

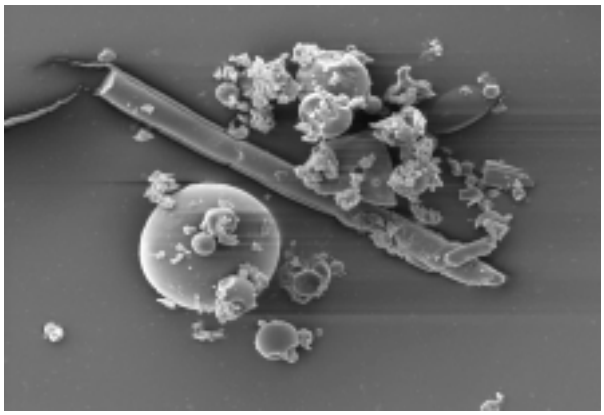
Table I. ^{13}C chemical shifts (ppm) of carbons of hyodeoxycholic acid (HDCA) in the absence and in the presence of 2-hydroxypropyl-β-cyclodextrin (HPβCD)

Carbon	δ HDCA	δ HDCA/HPβCD	$\Delta\delta$[a]
24	178.39	178.37	−0.02
23	32.30	32.23	−0.07
22	32.59	32.74	+0.15
21	19.05	19.62	+0.57
20	36.99	36.99	0.00
19	24.37	24.31	−0.06
18	12.77	13.16	+0.39
17	57.75	57.63	−0.12
16	29.45	29.81	+0.36
15	25.58	25.65	+0.07
14	57.93	58.09	+0.16
13	44.33	44.43	+0.10
12	41.66*	41.92	+0.26
11	22.21	22.18	−0.03
10	37.24	37.21	−0.03
9	41.62*	41.66	+0.04
8	36.51	36.53	+0.02
7	35.88	35.85	−0.03
6	68.96	69.00	+0.04
5	50.19	50.18	−0.01
4	30.31	30.34	+0.03
3	72.70	72.79	+0.09
2	31.44	31.52	+0.08
1	37.11	37.17	+0.06

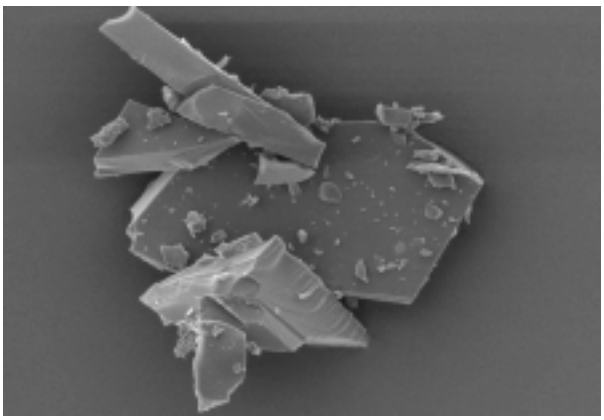
* The values may be interchanged.
[a] $\Delta\delta = \delta$ HDCA/HPβCD $-\delta$ HDCA.

The DSC curves revealed some information on the solid state interactions of HDCA with HPβCD (Figure 6). The HDCA crystals showed the characteristic endothermic peak at 204°C, corresponding to the bile acid melting. The DSC profile of HPβCD exhibited a broad endothermic peak (between about 60°C and 140°C) due to water loss. The appearance of two endothermic peaks corresponding to drug melting and to dehydration of HPβCD was also evident in the thermogram of the physical mixture. The HDCA melting peak disappeared in the thermograms of both solid inclusion complexes (evaporated inclusion complex and lyophilised inclusion complex).

(a)



(b)



(c)

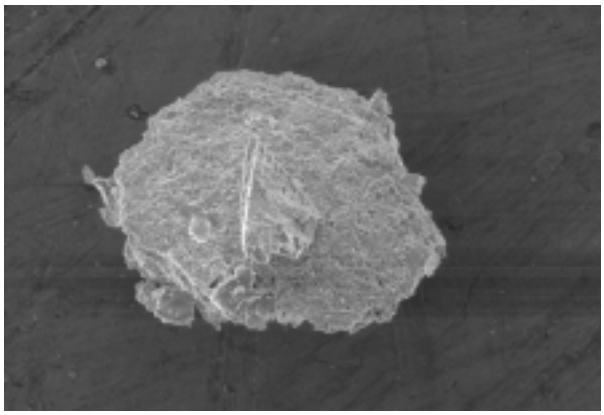


Figure 5. Scanning electron microphotographs. (a) physical mixture; (b) evaporated inclusion complex; (c) lyophilised inclusion complex.

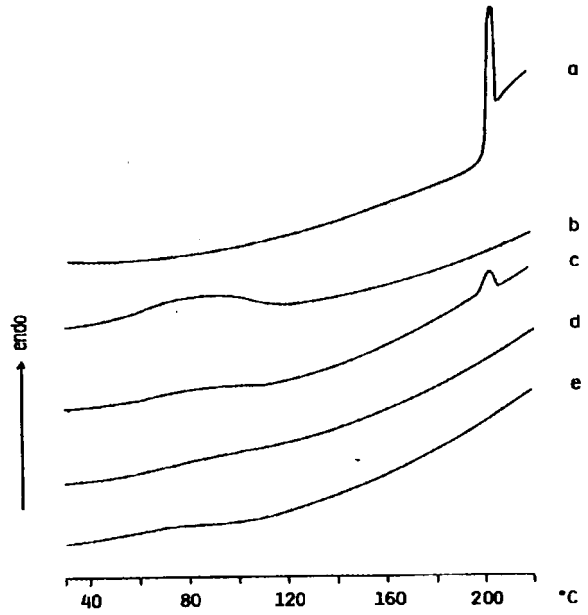


Figure 6. Differential scanning calorimetry (DSC) curves. (a) hyodeoxycholic acid; (b) 2-hydroxypropyl-β-cyclodextrin; (c) physical mixture; (d) evaporated inclusion complex; (e) lyophilised inclusion complex.

The X-ray pattern (Figure 7) of the physical mixture was the superimposition of the HDCA and amorphous HPβCD patterns. The X-ray diffraction patterns of both the lyophilised and evaporated inclusion complexes were typical of an amorphous inclusion complex.

The absence of the drug melting peak in the DSC thermogram and of interferences in the X-ray diffraction pattern of the inclusion complexes was attributed to the inclusion of the drug within the cyclodextrin cavity [26]. Clearly, the freeze-drying process of a substance not included in cyclodextrin should produce drug amorphisation. As the amorphisation of the drug alone or in a physical mixture can produce the same thermal behaviour (absence of the drug melting peak) and the same flat X-ray diffraction pattern of the amorphous complex, the X-ray and DSC data cannot confirm the formation of an inclusion complex by the freeze-drying process. However, the X-ray and DSC of the evaporated inclusion complex are clear evidence of the inclusion of HDCA into the cyclodextrin cavity. In fact, the evaporation of a HDCA solution without HPβCD does not produce the drug amorphisation.

The IR spectroscopic technique (Figure 8) was found to be suitable for detecting the inclusion of bile acid (UDCA) within the HPβCD cavity [12, 13]. The IR spectra of the physical mixture showed the typical bands of the two components (HCDA and HPβCD). Particularly evident is the broad band of the -OH groups

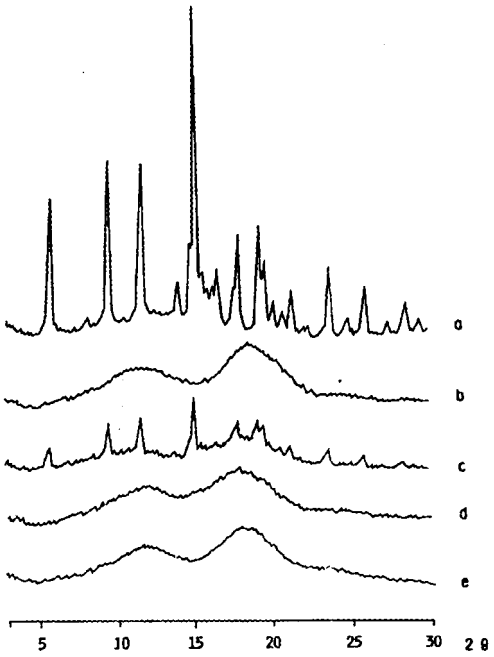


Figure 7. X-ray diffraction patterns. (a) hyodeoxycholic acid; (b) 2-hydroxypropyl-β-cyclodextrin; (c) physical mixture; (d) evaporated inclusion complex; (e) lyophilised inclusion complex.

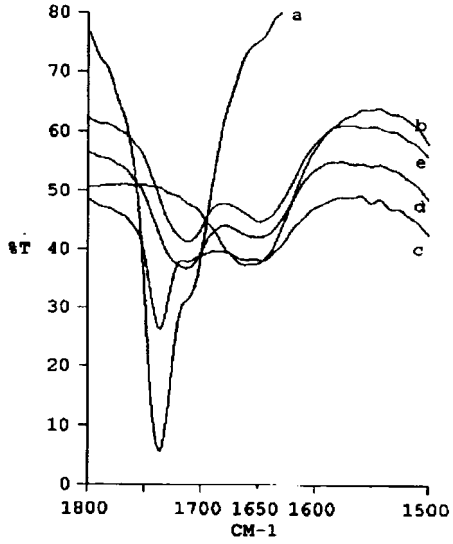


Figure 8. Infrared absorption bands in the 1800–1500 cm^{-1} region. (a) hyodeoxycholic acid; (b) 2-hydroxypropyl-β-cyclodextrin; (c) physical mixture; (d) evaporated inclusion complex; (e) lyophilised inclusion complex.

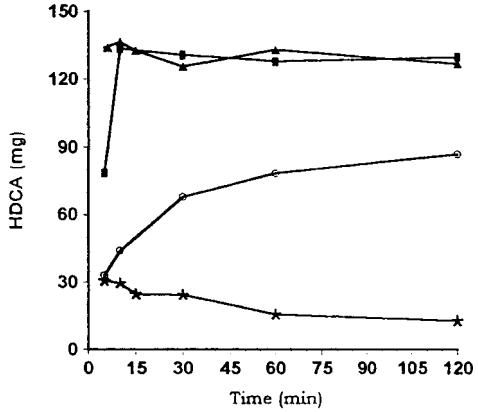


Figure 9. Dissolution profiles in water at 37 ± 0.1°C. ∗ hyodeoxycholic acid; ○ physical mixture; ▲ evaporated inclusion complex; ■ lyophilised inclusion complex.

of the cyclodextrin (3300–3400 cm^{-1}) which is superimposed on the band of the free OH group of HDCA (3436 cm^{-1}). Moreover the ν (C=O) stretching mode of HDCA (1736 cm^{-1}) is also present. This band is typical of HDCA only as the other bile acid showed the ν (C=O) stretching mode in the range between 1700–1717 cm^{-1} owing to the dimeric association [27]. The HPβCD bands changed as a consequence of the complex formation. In the spectrum of the solid inclusion complex, the ν (C=O) stretching mode of HDCA was shifted from 1736 to 1712 cm^{-1} regardless of the preparation method (freeze-drying or evaporation process). The shape of the C=O band was broader and less intense in the spectrum of the complexes than in that of the physical mixture, whereas both HDCA and the physical mixture had similar C=O band profiles. A similar finding was reported for the inclusion complex of UDCA with HPβCD [14].

3.3. DISSOLUTION STUDIES

Figure 9 shows the dissolution profiles of plain HDCA crystals, the physical mixture and the evaporated and lyophilised inclusion complexes with HPβCD.

The dissolution rate of the HDCA crystal is very slow and the bile acid reaches a maximum concentration value in the solution in 5 min before approaching a lower concentration. Up to now, it is not possible to explain the behaviour of HDCA during the dissolution tests. Further studies are planned to clarify the reasons for the decrease of the bile acid concentration during the dissolution tests.

The dissolution of the drug from the physical mixture was faster than that of the plain drug crystals, owing to the increase in the wettability of the drug crystals. In fact, the hydrophilic properties of HDCA crystals are improved by HPβCD particles that coat their surface. On the other hand, the dissolved HDCA molecules can interact with the HPβCD forming the inclusion complex in the dissolution medium. Besides the increase in the drug dissolution rate and solubility,

the dissolution profiles of HDCA from the physical mixture did not show any drug precipitation. As it is reasonable to suppose that the complex is formed during the dissolution, the complexation increases also the HDCA stability in solution.

The dissolution rate of HDCA from the complex was higher than that obtained for the plain drug crystals and physical mixture. The improvement of the dissolution characteristics can be justified through the concurrence of different factors. The inclusion of the HDCA molecules into the HPβCD cavity improves the wettability owing to the hydrophilicity of the exterior surface of the cyclodextrin derivative. The inclusion in the cavity of the highly soluble cyclodextrin derivative and the subsequent drug amorphisation can also explain the enhanced dissolution rate of the inclusion complex.

It is interesting to note that the dissolution rate of HDCA from the evaporated inclusion complex is faster than from the lyophilised inclusion complex in the first 5 min. In our opinion, this behaviour can be due to the greater aggregated structure of the lyophilised inclusion complex with respect to that of the evaporated inclusion complex, as the SEM microphotographs showed (Figures 5b and 5c).

4. Conclusion

The complexation of HDCA into the HPβCD cavity was suggested by study in the solid state and in solution. The value of the apparent stability constant of the 1 : 1 inclusion complex (K) (1820 $\pm$ 178 M^{-1}) is within the range 200–5000 M^{-1} considered as adequate by several authors to improve the bioavailability of poorly soluble drugs [28, 29]. The solid inclusion complex showed an increase both in solubility and in dissolution rate with respect to the HDCA crystals alone or in a physical mixture, with the dissolution rate of HDCA from the evaporated inclusion complex being faster than from the lyophilised inclusion complex. Therefore, both the K value and the enhanced dissolution rate offers the promise of increased HDCA bioavailability. Hence, the above positive results motivate "in vivo" bioavailability studies to evaluate the feasibility of the complex in oral administration of HDCA. According to the results of the dissolution experiments and to the less expensive procedure, the evaporated inclusion complex will be employed for the formulation development.

The inclusion procedure is now available to evaluate its capability to induce a rise of HDCA in the biliary pool in comparison with the plain bile acid.

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